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Full Length Article

Overexpression of *SOC1-Like* Gene Promotes Flowering and Decreases Seed Set in *Brachypodium*

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Abstract

MADS-box genes play important roles in many aspects of plant growth and development. It has been suggested that *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*)*-like* MADS-box genes function as activators of floral transition in *Brachypodium distachyon*. In this study, Bioinformatics analysis suggested the presence of a conservative MADS-box and keratin (K) domain that belong to the *SOC1* family. The expression of *Brachypodium SOC1-like (BdSOC1-like)* was widely detected in different tissues/organs of *Brachypodium*, including roots, stems, young leaves, old leaves, shoot apical meristem (SAM) and flowers at different developmental stage. Also, quantitative real-time (qRT) PCR results suggested that the accumulation of *BdSOC1-like* gene did not display a diurnal rhythm under long-day (LD, 18-h light/6-h dark) or short-day conditions (SD, 10-h light/14-h dark) and its expression did not appear to remember the prolonged cold treatment in *Brachypodium*. The heading date was earlier in plants overexpressing *BdSOC1-like*, which led to a reduced seed set in *Brachypodium*. In combination, above results suggest that *BdSOC1-like* may play important roles in the transition to flowering in *Brachypodium*. © 2019 Friends Science Publishers

Keywords: *Brachypodium*; MADS-box; *BdSOC1-like*; Overexpression; Flowering

Introduction

The shift from vegetative growth to inflorescence meristem identity signifies the beginning of the reproductive growth phase and is a key determinant of flowering time. In order to optimize sexual reproduction and productivity, plants accurately control the timing of shift from vegetative growth to inflorescence meristem identity (Putterill *et al*., 2004). Many studies on flowering time have been reported in many species (Lee *et al*., 2007; Vain *et al*., 2008; Kim *et al*., 2009; Srikanth and Schmid, 2011; Tamura *et al*., 2011; Andres and Coupland, 2012) and are best understood in *Arabidopsis thaliana*. Both exogenous cues (such as day length and temperature) and endogenous factors (such as developmental status and age) impact the shift from vegetative growth to inflorescence meristem identity in plants (Simpson and Dean, 2002; Boss *et al*., 2004; Lee *et al*., 2007). In *A. thaliana*, six major pathways (photoperiod, vernalization, thermosensory pathways, autonomous, age and gibberellin pathways) influence flowering time in response to those exogenous and endogenous signals (Wahl *et al*., 2013). The six pathways involved many integrator genes (such as *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1/AGAMOUS-LIKE 20* (*SOC1/AGL20*) and *SHORT VEGETATIVE PHASE* (*SVP*)), which either rapidly promote or prevent the shift from vegetative growth to inflorescence meristem identity in *A. thaliana* (Lee *et al*., 2000; Lee and Lee, 2010).

In *A. thaliana*, *SOC1* has been involved in floral transition and development. *SOC1* is a member of the MADS-box transcription factor family and it consists of the MADS-box, keratin (K) box, C-terminal domain and SOC1 motif (Lee *et al*., 2004; Zhong *et al*., 2012). The *SOC1* gene encodes a MADS-box transcription factor that functions in the maintenance of vegetative shoot identity and promotes floral transition (Liu *et al*., 2009). The *SOC1* gene is differentially expressed in the roots, stems, leaves, shoot apical meristem and floral organs (Borner *et al*., 2000; Watson and Brill, 2004; Shen *et al*., 2011). In addition, the expression levels of *SOC1* are often regulated by multiple flowering pathways (Shen *et al*., 2011). As a floral integrator, *SOC1* is controlled by *FLC* and CONSTANS (*CO*), which acts as a floral activator and activates *SOC1 via* the *FT* gene in response to photoperiod (Hepworth *et al*., 2002; Yoo *et al*., 2005). *FLC* and *SVP* act as floral transition repressors, and their interaction downregulate the expression of *SOC1* in whole plants and *FT* in the leaves (Searle *et al*., 2006; Li *et al*., 2008). During the transition from vegetative growth to reproductive growth, the

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transcription of *FLC* and *SVP* is repressed by stimulatory flowering signals, which promote the expression of *FT* and *SOC1* (Lee *et al*., 2007; Li *et al*., 2008).

Flowering time has also been widely investigated in crops (Kim *et al*., 2009; Turck *et al*., 2008; Lagercrantz, 2009). For instance, the tropical cereal rice (*Oryza sativa*) has homologues of many flowering-time genes defined in *A. thaliana* (Colasanti and Coneva, 2009). In addition, both photoperiod and autonomous pathways are similar between *O. sativa* and *A. thaliana* (Dennis and Peacock, 2009). To elucidate the genetic control of flowering time, many studies have been also performed in wheat, which also has photoperiod sensitivity, vernalization requirement and autonomous flowering pathway (reviewed in Worland and Snape, 2001). The photoperiod (long-day) sensitivity gene, *Ppd-A1*, *Ppd-B1* and *Ppd-D1*, have been genetically identified on chromosome2A, 2B and 2D, respectively. Vernalization requirement is determined by dominant genes *Vrn-A1*, *Vrn-B1* and *Vrn-D1*, which control insensitivity to vernalization. So far, no major genes have been characterized for autonomous flowering pathway. *Brachypodium* is a model for the temperate grasses including barley and wheat (Bossolini *et al*., 2007; International *Brachypodium* Initiative, 2010). Compared with the model dicot *A. thaliana* and monocot *O. sativa*, in aspects of photoperiod pathway and autonomous pathway, *Brachypodium* has homologues of genes defined in *A. thaliana*. With the exception of *VRN2* which is absent in *Brachypodium*, it has homologues of vernalization pathway genes defined in cereals (Distelfeld *et al*., 2009; Higgins *et al*., 2010). CO3 is also absent in *Brachypodium* (Higgins *et al*., 2010). In addition, experimental studies of *SOC1-like* genes from rice and wheat have been also reported and results have shown that although the wheat SOC*1-like* can partially restore the phenotype of an *Arabidopsis soc1* mutation, its transcription was not in response to photoperiod or vernalization (Shitsukawa *et al*., 2007). However, the structure, expression pattern, and role of the homologous *SOC1-like* gene are not well understood in *Brachypodium*.

To investigate the roles of MADS-box genes in *Brachypodium*, we identified and characterized a *Brachypodium SOC1-like* gene (*BdSOC1-like*) by analyzing its expression patterns, and investigated the potential roles of *BdSOC1-like* gene in the photoperiod and vernalization response of *Brachypodium*. In addition, functional analysis of the *BdSOC1-like* gene was conducted by overexpressing this gene in *Brachypodium*. Our results facilitate a better understanding the role of *SOC1-like* gene during the shift from vegetative growth to inflorescence meristem identity in winter cereal crops.

Materials and Methods

Plant Materials and Growth Conditions

In our experiments, unless otherwise noted, all seeds derived from the *B. distachyon* ecotype Bd21 were placed on two filter papers in a petri dish (9 cm in diameter) with 5 mL water in the dark at 4°C for 7 d to break the dormancy (An *et al*., 2015). Seedlings were then transplanted into the soil for subsequent analysis. All seedlings were grown in a greenhouse under long-day (LD, 16-h light/8-h dark) or short-day conditions (SD, 10-h light/14-h dark) unless stated elsewhere. The average temperature was 19°C.

Plasmid Construction

Full-length open reading frames (ORFs) of *BdSOC1-like* (Gene Bank ID: Bradi3g32090.1) were amplified *via* specific primers (Table S1) and cloned into a pMD19-T vector (TaKaRa, Dalian, China, 1068A). The fragments were digested with *Kpn*I and *Hin*dIII and purpose fragments were subcloned into $pPZP^{211}$ carrying the ZmUbi promoter.

Bioinformatics Analysis

Both the nucleotide sequence and the amino acid sequence of *SOC1-like* derived from selected species were assayed through NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The domain of *BdSOC1-like* sequence was deduced using the motif scan analysis (http://myhits.isb-sib.ch/cgi-bin/motif_scan/). Sequence alignment of SOC1 protein from various species were aligned by ClustalW (Thompson *et al*., 1994). The amino acid sequences of *BdSOC1-like* and other *SOC1* genes were used to constructed a phylogenetic tree through Neighbor-Joining (NJ) method (Saitou and Nei, 1987).

qRT-PCR Analysis

Total RNA was isolated from the whole plants of *Brachypodium* harvested at the zeitgeber time (ZT8) and extracted using Trizol (Invitrogen, Carlsbad, U.S.A.). Complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using reverse transcription kit (Bioteke, Beijing, China) in a total volume of 20 μL. qRT-PCR was performed on a Bio-Rad (Hercules, CA, USA) CFX96 with SYBR Green Master Mix (CWBIO, Beijing, China, CW0760). The transcription levels of the relative flowering time genes were assayed by qRT-PCR. The ACTIN gene and UBC18 were used as housekeeping controls. The primers of the relative genes are listed in Table S3. The qRT-PCR reaction contained 25 μL 2× UltraSYBR Mixture, 1 μL forward primer (10 m*M*), 1 μL reverse primer (10 m*M*) and 1 μL of 1:10 diluted cDNAs, made up to a total volume of 50 μL with ultrapure water. The qRT-PCR was performed under the following conditions: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and then 60°C for 1 min. The results were analyzed using the relative $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Three technical repeats were carried out for each cDNA sample. Three biological repeats (sample repeats, sample RNA derived

from different plants growing under the same conditions) were carried out.

Genetic Transformation

To induce embryogenic calli, immature embryos were placed on induction medium for 6 weeks in Bd21. The calli were co-cultivated with *Agrobacterium* strain *EHA105* on Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) solid medium with 80 mg/L of G418 and cultured at 28°C in the absence of light for 24 d. The resistant calli were transferred to regeneration medium with same antibiotics for plant regeneration under 16:8 h light-dark at 26°C. After 2–3 weeks healthy shoots appeared and were transferred onto rooting medium. Plants with healthy roots were analyzed with PCR and reverse transcription PCR (RT-PCR). Transgenic plants were those that carried *NPTII* and *Ubi-BdSOC1-like* and were then transferred into soil. The procedures described above were based on the protocols of Alves *et al.* (2009).

PCR and RT-PCR

DNA and RNA from leaves of 11 putative T_0 transgenic Bd21 lines harvested at the ZT8 were subjected to molecular analyses to demonstrate the presence and transcription levels of the *NPTII* and *Ubi-BdSOC1-like* genes. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) and complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using reverse transcription kit (Bioteke, Beijing, China) in a total volume of 20 μL. For PCR, we used 1 μL of DNA solution and primers at a final reaction concentration of 0.4 μM. For RT-PCR, we used 1 μL of cDNA solution and primers at a final reaction concentration of 0.4 μM. The primers used to amplify the *NPTII* (525 bp) *Ubi-BdSOC1-like* (684 bp) and *BdUBC18* (500 bp) genes were listed in Table S3. The annealing temperature for both genes was 58°C and the products were assayed with agarose gel electrophoresis.

Inheritance of the Transgene in Progeny

Forty to sixty randomly chosen grains from wild-type (WT) or 11 independent T_1 transgenic lines were placed on two filter papers in a petri dish (9 cm in diameter) with 5 mL water. Plates were kept in a growth incubator (4°C, dark) for 4 d. Then, the grains were transferred to the Petri dish (9 cm in diameter) with two filter papers and 80 mg/L G418. The plates were incubated for 4 days under continuous light at 23°C. To demonstrate the presence of the *NPTII* and *Ubi-BdSOC1-like* genes, DNA from putative transgenic Bd21 plants with white roots was extracted using CTAB method (Doyle and Doyle, 1987). T_1 seedlings with white roots were identified and the segregation of transgene in the T_1 population was statistically analyzed for the expected Mendelian ratio of 3:1 of a single locus by Chi-square (χ^2) test.

Flowering time Measurement

Flowering time, represented by days to heading and total leaf numbers, was analyzed in plants grown from germinated seeds. In our experiments, the flowering time of the WT and 11 transgenic T_0 plants was measured as the time from the budding stage of plant genetic transformation to flowering. In the T_1 generation, the time from seeds germination to heading and the number of primary leaves derived from the main shoot (>8 plants) was used to determine the time of the plants heading. Three biological repeats (sample repeats, all phenotypic data was measured from plants grown under LD conditions and these plants were developed from different T_1 seeds) were performed in the experiment of flowering time measurement.

Grain Trait Measurements

Fully-filled grains from WT or 3 representative T_1 transgenic lines (*BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7*) were used for measuring 1-grain length (mm), 20-grain length (cm), 1-grain width (mm), 20-grain width (cm) and 1,000-grain weight (g). Thirty randomly selected grains for WT or representative T_1 transgenic plants were used to measure the 1-grain length and 1-width using Image J software (Chen *et al*., 2016). Four hundred randomly selected grains for WT or representative T_1 transgenic plants, each group consisting of 20 grains, were used to measure the 20-grain length and width using Image J software (Chen *et al*., 2016). One thousand randomly chosen grains from WT or representative T_1 transgenic lines were used to measure grain weight. Average seed production per plant was also counted. In addition, to Investigation of seed germination rate in the T1 generation, thirty randomly chosen grains from wild-type (WT) or 11 independent T_1 transgenic lines were placed on two filter papers in a petri dish (9 cm in diameter) with 5 mL water. Plates were kept in a growth incubator (4°C, dark) for 4 d and then count the number of germinated and ungerminated seeds. Three technical repetitions and three biological repeats (sample repeat, all phenotypic data was measured from different T_1 grains) were performed.

Statistical Analysis

In this study, at least three technical and biological repeats were done separately for each experiment. The statistical method in Wu *et al*. (2014) was used for analyses of the *P*-value of the data.

Results

Molecular Cloning and Bioinformatics Analysis of *BdSOC1-like*

The *SOC1-like* gene was isolated from *Brachypodium* and named *BdSOC1-like* (Bradi3g32090.1). The *BdSOC1-like* encodes an ORF of 684 bp with a predicted protein of 227 amino acids. Compared to other *SOC1* or *SOC1-like* genes from *Arabidopsis*, wheat, rice and *Camelina*, the *BdSOC1-like* gene encodes MADS-domain protein with 59.4% amino acids identity to each other. Alignment with other *SOC1-like* proteins indicated that the MADS-box (91.93% identity), K- box, C-terminal domain and SOC1 motif were well conserved in *BdSOC1-like* (Fig. 1a). The *BdSOC1-like* gene contains seven introns, and the gene is bigger as a result of having a larger second intron. The phylogenetic tree suggested that *BdSOC1-like* was most related to *OsMADS56*, which was consistent with Higgins *et al*. (2010) (Fig. 1b).

Differential Spatial Expression of *BdSOC1-like*

To understand the potential roles of *BdSOC1-like*, the different tissues or organs from WT pants involving roots, stems, young leaves, old leaves, SAM and flowers were harvested at the ZT8 at the 1-leaf, 4-leaf and 7-leaf stages after cultivating the seedlings under LD conditions (18-h light/6-h dark), respectively. We examined the spatiotemporal pattern of *BdSOC1-like* mRNA accumulation by qRT-PCR under LD conditions. Under the same conditions, *BdSOC1-like* was diffusely expressed in various organs or tissues, including the roots, stems, young leaves, old leaves, SAM and flowers. Its expression in roots, leaves, and flowers was higher, and was less than that in stem at the 1-leaf, 4-leaf and 7-leaf stages. Besides, expression of *BdSOC1-LIKE* is less at the 1-leaf, 4-leaf stages (Fig. 2a). As previously reported in *Arabidopsis* (Lee and Lee, 2010), the *BdSOC1-like* transcripts were also mainly detected in the roots, leaves and flowers in the vegetative stage and the reproductive stage. This implied that *BdSOC1-like* might be involved in the regulation of flowering time in *Brachypodium*.

The Expression of *BdSOC1-like* did not Display a Diurnal Rhythm

Day length or day/night cycle influences the transition to flowering in plants through the photoperiod pathway (Imaizumi and Kay, 2006). It has been reported that the expression of *SOC1-like* is not controlled by photoperiod or vernalization in rice and wheat (Shitsukawa *et al*., 2007). To confirm whether day/night rhythm could influence the expression of *BdSOC1-like*, whole seedlings of WT plants grown for 10 d were harvested directly at 4-h intervals

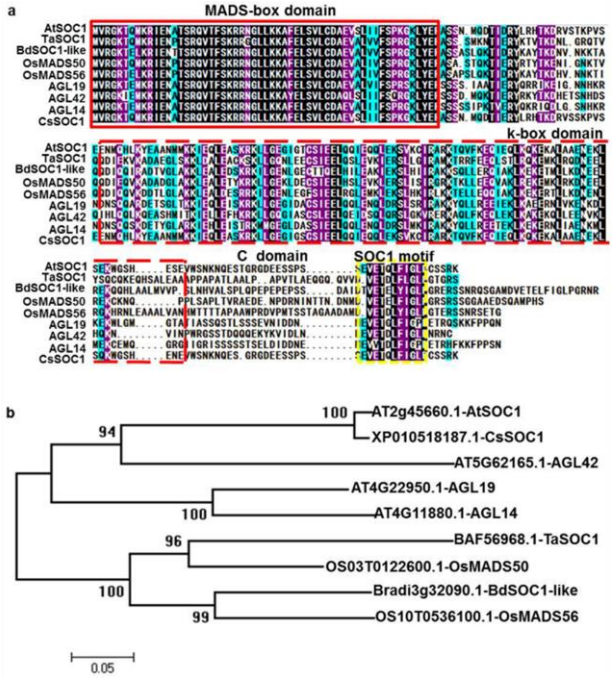

Fig. 1: Phylogenetic relationship of *BdSOC1-like* and related MADS-box genes from other species

(a) The proteins were aligned using ClustalW. The accession numbers of the genes are *AtSOC1* (AT2g45660), *TaSOC1* (BAF56968), *BdSOC1-like* (Bradi3g32090), *OsMADS50* (OS03T0122600), *OsMADS56* (OS10T0536100), *AGL19* (AT4G22950), *AGL42* (AT5G62165), *AGL14* (AT4G11880), and Cs*SOC1* (XP010518187). (b) Neighbor-joining tree of TARGET OF *SOC1-like* and related proteins. Sequences based on the amino acid alignment of BdSOC1-like proteins and SOC1-like proteins from other species. Sequences of *BdSOC1-like* of *Brachypodium* (Bradi3g32090.1), *SOC1*, *AGL19*, *AGL42*, and *AGL14* of *Arabidopsis* (AT2g45660.1, AT4G22950.1, AT5G62165.1, AT4G11880.1), *SOC1* of wheat (BAF56968.1), *OsMADS50* and *OsMADS56* of rice (OS03T0122600.1, OS10T0536100.1), and *SOC1* of *Camelina* (XP010518187.1) were used. The scale, observed divergency

throughout a 72-h cycle under LD (18-h light/6-h dark) or SD (10-h light/14-h dark) conditions, and the expression levels of *BdSOC1-like* were examined *via* qRT-PCR. The qRT-PCR analysis showed that transcription level of *BdSOC1-like* has no obvious regularity between day and night during both LD and SD conditions (Fig. 2b). The results implied that the accumulation of *BdSOC1-like* gene did not display a diurnal rhythm under LD or SD conditions in *Brachypodium.*

Impact of Cold Treatment on the Expression of *BdSOC1-like*

To investigate the impact of the cold treatment, WT seedlings were transferred into soil and placed at 4°C (10-h light/14-h dark) for 6 weeks and then moved to greenhouse at 19°C for 2 weeks. Non-vernalized control plants were grown in greenhouse at 19°C until they reached a developmental stage which was equivalent to that of the vernalized plants at the time of harvesting. The whole plants were harvested at the ZT8. We examined the expression of the *BdSOC1-like* gene during and after cold treatment by

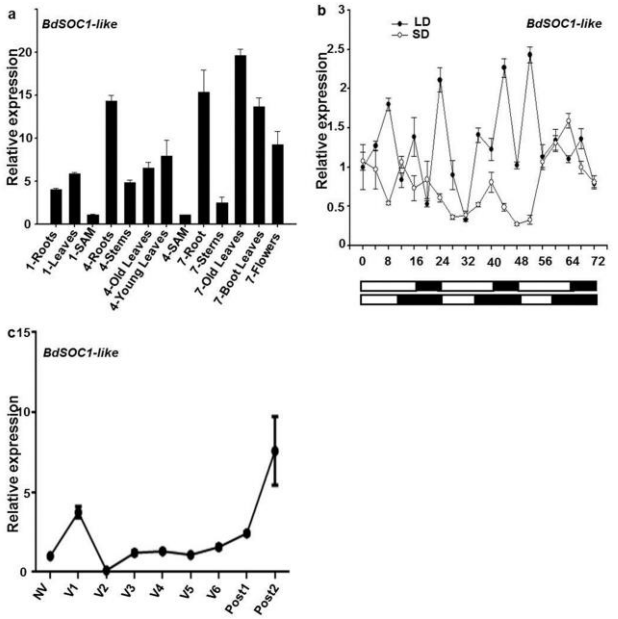

Fig. 2: Developmental regulation of *BdSOC1-like* during LD and SD or the course of vernalization

(**a**) The spatial expression of *BdSOC1-like* in the different tissues of Bd21.The relative expression of *BdSOC1-like* in the different tissues or organs from WT plants was detected by qRT-PCR at the 1-leaf, 4-leaf, and 7-leaf stages after cultivating the seedlings under LD conditions (18-h light/6-h dark). The roots, stems, young leaves, old leaves, SAM, and flowers were harvested at the ZT8. (**b**) Diurnal and circadian expression of *BdSOC1-like*. The black line indicates LD conditions (18-h light/6-h dark) and the gray line indicates SD conditions (10-h light/14-h dark). Transcript accumulation was measured at 4-h intervals throughout a 72 h cycle using qRT-PCR analysis of specific genes, and normalized to *BdUBC18*. Data was measured from whole seedlings of WT plants grown for 10 d, which were harvested directly at 4-h intervals throughout a 72 h cycle under LD (18-h light/6-h dark) or SD (10-h light/14-h dark) conditions. (**c**) mRNA expression analysis of *BdSOC1-like* during the course of vernalization using qRT-PCR. NV, non-vernalized; V1, one week of vernalization; V2, two weeks of vernalization; V3, three weeks of vernalization; V4, four weeks of vernalization; V5, five weeks of vernalization; V6, six weeks of vernalization; Post1, six weeks of vernalization followed by one week of normal growth temperature; Post2, six weeks of vernalization followed by two weeks of normal growth temperature. Data was measured from whole seedlings of WT plants harvested at the ZT8. Relative fold change was determined by normalization with *BdUBC18* levels. *BdUBC18* was used as a control gene. Error bars denote the s.e.m. of three technical repetitions each. Three biological replications were performed and the expression trends of *BdSOC1-like* were similar

qRT-PCR. *BdSOC1-like* transcript dramatically increased in the first week of cold exposure, then decreased in the second week of vernalization, and then steadily increased thereafter (Fig. 2c). These results suggested that the expression of *BdSOC1-like* did not appear to remember the prolonged cold treatment in *Brachypodium.*

Regeneration and Preliminary Analysis of T_0 Transgenic Plants

In the genetic transformation experiment, we obtained 24 putative T_0 transgenic plants. Unfortunately, only 11 putative transgenic lines had the T_1 seeds, the rest of the lines did not obtain T_1 seeds due to premature flowering. To determine the presence and expression of the foreign genes, the 11 putative T_0 transgenic plants were chosen for molecular analysis. First of all, to confirm the presence of *NPTII and Ubi-BdSOC1-like*, total DNA from leaves of 11 putative T_0 transgenic plants was extracted and analyzed by PCR in putative transgenic plants. Secondly, total RNA from leaves of 11 putative T_0 transgenic plants was extracted and analyzed by RT-PCR to evaluate the expression of *NPTII* and *Ubi-BdSOC1-like* in putativeT_0 transgenic plants. As shown in Fig. S1, relative bands of the two genes could be expanded by both PCR and RT-PCR in putativeT_0 transgenic plants. These results suggested that the purposed genes could be integrated into the *Brachypodium* genome and were stably expressed in the putative transgenic plants.

Assay of Segregation of Transgene in T_1 Transgenic Plants

To ensure that we could get seeds from the putative T_0 transgenic plants, T_1 progeny of 11 independent T_0 transgenic plants were chosen to evaluate the inheritance patterns of the *NPTII* transgene. The T_1 seedlings were treated with G418 for 4 days and then observing the color change of the root under room condition. We found that the roots of putative transgenic seedlings did not change, and the roots from non-tansgenic seedlings were black (Fig. S2a). The results showed that white roots were resistant to G418 and therefore transgenic, whereas black roots were sensitive to G418 and non-transgenic. In addition, to confirm the presence of *NPTII* in the putative T_1 transgenic plants, the total DNA from leaves of the seedlings with white roots was extracted and analyzed by PCR. As shown in Fig. S2b, relative bands of *NPTII* could be expanded in the putative T_1 transgenic seedlings with white roots. The result implied that the seedlings with white roots were transgenic. Furthermore, the expected segregation ratio should be 3:1 ($NPTII^+$ versus $NPTII^-$) if the T_0 plants were self-pollinated and there was only one copy of *NPTII* transgene existing in the Bd21 genome. Actually, we found that only the progeny of one transgenic line gave large bias ($\chi 2 \geq 3.41$), which suggested the existence of multiple transgene copies in the Bd21 genome, and the others were in accordance with 3:1 Mendel's law (Table S1).

The Overexpression of *BdSOC1-like* Promoted the Transition from Vegetative to Reproductive Growth

SOC1 has been isolated from other species and their function characterized as integrators are conservative during the transition period (Watson and Brill, 2004; Shitsukawa *et al*., 2007; Lee and Lee, 2010; Wei *et al*., 2016). To understand the potential function of the *BdSOC1-like* gene in flowering time control, the flowering time was assayed using the T_0 or T_1 plants of 11 transgenic lines.

In the T_0 generation, under LD conditions, the WT flowered for 90 d on average, while the transgenic plants flowered for approximately 50 days on average. The results indicated that *BdSOC1-like* might play an active role during the shift from vegetative growth to inflorescence meristem

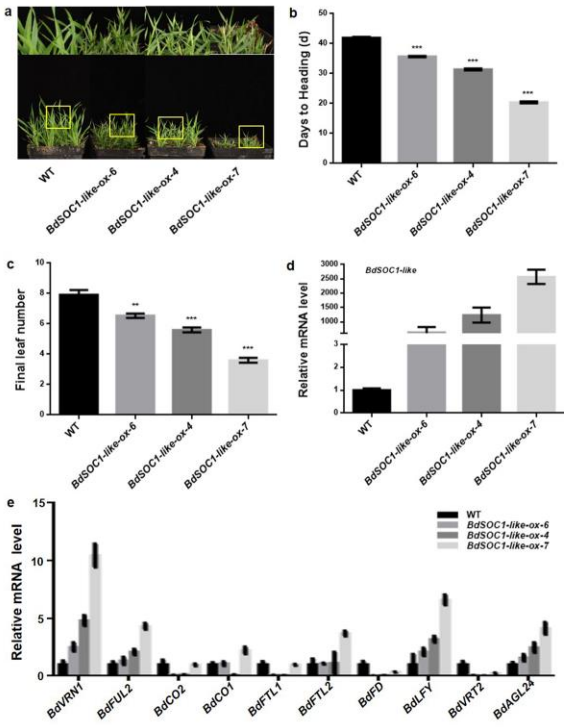

Fig. 3: Influence of *BdSOC1-like* on flowering in the T_1 generation

(**a**) Representative plants illustrating the phenotypic difference between the WT and early-flowering plants. (**b**, **c**) Flowering time of WT and representative transgenic lines (*BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7*). The flowering time of the plants was measured as the days to heading or the number of primary leaves derived from the main shoot after flowering. (**d**, **e**) Expression levels of *BdSOC1-like* or related flowering time genes in whole seedlings of representative transgenic plants (grey) relative to that of WT plants (black), grown for 10 d. All phenotypic data was measured from plants cultivated under LD conditions (16-h light/8-h dark). Data in b-c are shown as means ± s.d. of three technical repetitions (n=8-15). Data in d-e was shown as means ±s.e.m. of three technical repetitions. Student's *t*-tests were used to generate the *P*-values: *t*-test: **, $P < 0.01$; ***, $P < 0.001$. Three biological replications were performed and all results were similar

identity (Fig. S3a–c).

Then, in the T_1 generation, the representative transgenic lines (*BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7*) were selected to assess the relationship between transcription levels of related genes and phenotype in WT and transgenic lines under LD conditions. Compared to WT plants, the *BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7* lines flowered ahead of time (Fig. 3a). The WT plants flowered for 42 d on average (7–8 leaves), *BdSOC1-like-ox-6* flowered for 36 d on average (6–7 leaves), *BdSOC1-like-ox-4* flowered for 31 d on average (5-6 leaves), and *BdSOC1-like-ox-7* flowered for 21 d on average (3–4 leaves) (Fig. 3b–c). Correspondingly, the mRNA levels of *BdSOC1-like* were much higher in *BdSOC1-like-ox-7* than that in WT, *BdSOC1-like-ox-6* and *BdSOC1-like-ox-4* and were proportional to the flowering time (Fig. 3d). The other transgenic lines also exhibited advanced flowering time, ranging from 16 to 35 d on average (3–6 leaves) (Table 1 and Fig. S4a–b). Correspondingly, the mRNA levels of *BdSOC1-like* were consistent with corresponding phenotype in WT and other transgenic lines (Fig. S4d). Furthermore, in *Brachypodium*, qRT-PCR of the transgenic lines suggested that some relative flowering genes (such as *BdVRN1*, *BdLFY*, and *BdAGL24*) were promoted, while some others (such as *BdFTL1*, *BdFTL2*, *BdCO1*, and *BdCO2*) were not (Fig. 3e).

The above results suggested that *BdSOC1-like* could positively influence the floral transition process by promoting key flowering-related genes, such as *BdVRN1* and *BdLFY, in B. distachyon.*

Table 1: Flowering time analysis of and *BdSOC1-like-ox* in the T_1 generation under LD conditions

Genetic Background		DTH	LN	No. of T_1 plants
BdSOC1-like-ox	Bd21	42.00 ± 0.36	7.92 ± 0.31	12
	#1	22.77 ± 0.41	4.08 ± 0.21	13
	#2	22.50 ± 0.57	3.88 ± 0.23	8
	#3	15.64 ± 0.39	3.27 ± 0.14	11
	#4	31.40 ± 0.31	5.60 ± 0.16	10
	#5	32.22 ± 0.32	5.33 ± 0.17	9
	#6	35.69 ± 0.21	6.54 ± 0.14	13
	#7	20.40 ± 0.34	3.60 ± 0.16	10
	#8	26.88 ± 0.30	5.25 ± 0.16	8
	#9	35.00 ± 0.29	6.63 ± 0.17	9
	#10	24.83 ± 0.47	4.50 ± 0.15	12
	#11	14.78 ± 0.40	3.33 ± 0.17	9

DTH: days to heading; LN: final number of leaves; No. of T_1 plants; In three biological replications, the data were similar

The Overexpression of *BdSOC1-like* in *Brachypodium* Led to Pleiotropic Phenotypes

Fruit quantity and quality are important characteristics in all crops and are controlled by various internal factors and external environments. We obtained transgenic plants with *BdSOC1-like* being overexpressed and controlled by the ZmUbi promoter, which also showed a pleiotropic phenotype with abnormal seeds. To investigate the pleiotropic role of *BdSOC1-like*, the representative transgenic lines (*BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7*) were selected to assess the seed traits of the WT and transgenic lines under LD conditions. The 1-grain length, 20-grain length, 1-grain width, 20-grain width and 1,000-grain weight of the seeds and average seed production per plant were measured in the WT, *BdSOC1-like-ox-6*, *BdSOC1-like-ox-4*, and *BdSOC1-like-ox-7* lines during reproductive growth. As shown in Fig. 4a-f, the 1-grain and 20-grain length of the *BdSOC1-like-ox-7* line were ~20% less and its 1-grain and 20-grain width were ~30% less in comparison to WT. The 1-grain and 20-grain length of the *BdSOC1-like-ox-7* line were ~15% less and its 1-grain and 20-grain width were ~18% less in comparison to *BdSOC1-like-ox-6* line. The 1-grain and 20-grain length of the *BdSOC1-like-ox-7* line were ~6% less and its 1-grain and 20-grain width were ~10% less in comparison to *BdSOC1-like-ox-4* line. In addition, the 1,000-grain weight and average seed production per plant of representative transgenic lines were also less in comparison to WT (Fig. 4g-h). Correspondingly, the other transgenic lines also exhibited less seed production, ranging from 45 to 121 on

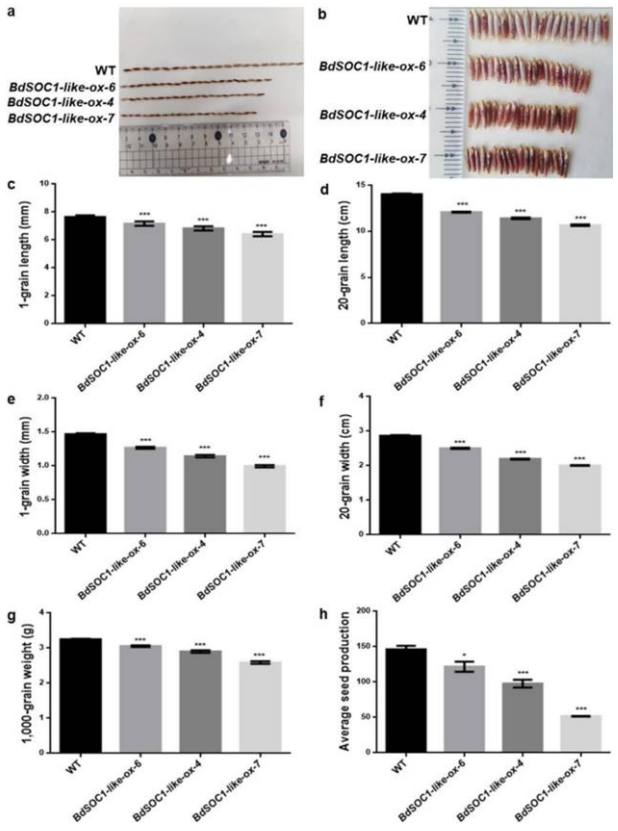

Fig. 4: The grain trial of WT and *BdSOC1-like-ox* transgenic plants

(**a**, **b**) Grains from representative plants illustrating the phenotypic difference between WT and *BdSOC1-like-ox* transgenic lines (*BdSOC1-like-ox-6*, *BdSOC1-like-ox-4* and *BdSOC1-like-ox-7*). (**c**) 1-grain length. (**d**) 20-grain length. (**e**) 1-grain width. (**f**) 20-grain width (**g**) 1,000-grain height. (**h**) Average seed production per plant. All phenotypic data was measured from plants grown under LD conditions (16-h light/8-h dark). Data in c-g was shown as means ± s.d. of three technical repetitions (c, e: n=30; d, f, g, h: n = 20). Student's *t*-tests were used to generate the *P*-values: *t*-test: ***, $P < 0.001$. Three biological replications were performed and all results were similar

average (Fig. S4c). These resulted in the formation of a smaller grain. In addition, compared with the wild type, the average germination rate of transgenic seeds was lower (Table S2). The above results suggested that a greater proportion of seeds from the *BdSOC1-like* transgenic lines were defective in comparison with those from the WT plants.

Discussion

MADS-box genes encode transcription factors related to various important biological functions in plants (Shore and Sharrocks, 1995). Molecular and genetic studies have reported that they have many important functions in aspects of plants growth and development (Ng and Yanofsky, 2001). In the term of flowering time, MADS-box transcription factors have been widely investigated in various plants and results suggests that they may be involved in the regulation of floral transition through some potential mechanisms (Theissen *et al*., 2000; Kater *et al*., 2006; Leseberg *et al*., 2006). The *Arabidopsis* SOC1, which is a typical MADS-box protein, regulates the shift from vegetative growth to inflorescence meristem identity. Many homologous *SOC1* or *SOC1-like* genes have been characterized in previous studies, and their roles have been also characterized in several other species (Borner *et al*., 2000; Zhong *et al*., 2012; Wei *et al*., 2016). Despite Bd21 being an important model plant for winter cereal crops and closely related to wheat, which is an economically important global food and forage crop, the molecular mechanisms of floral transition are largely unclear in *Brachypodium*.

In our study, *Brachypodium BdSOC1-like* gene was identified and its predicted proteins clustered within the MADS-box gene family regulators clade, which was associated with flowering time. Phylogenetic analysis suggested that *BdSOC1-like* might be orthologous to *AtSOC1* in thaliana, *TaSOC1* in wheat and *OsMADS50* and *56* in rice. Furthermore, sequence alignment with other *SOC1-like* proteins indicated that the MADS-box (91.93% identity), K-box, C-terminal domain, and SOC1 motif, which were typical characteristics of the MADS-box, were well conserved in the BdSOC1-like (Ruokolainen *et al*., 2011; Ding *et al*., 2013; Fig. 1). Based on the amino acid sequence analysis, *BdSOC1-like* was identified as a member of MADS-box family. In addition, *BdSOC1-like* was highly expressed in both vegetative and reproductive organ or tissues (Fig. 2a). This pattern was similar to that of *AtSOC1*, and was consistent with the possible function of the *BdSOC1-like* gene during the vegetative and reproductive phase. However, the accumulation of the *BdSOC1-like* gene did not display a diurnal rhythm under LD or SD conditions, and its expression did not appear to remember the prolonged cold treatment in *Brachypodium* (Fig. 2b–c), which were consistent with some previously reported *SOC1-like* genes from rice and wheat (Shitsukawa *et al*., 2007).

Moreover, in *A. thaliana*, *AtSOC1* is upregulated by *FT* and repressed by *FLC*. *AtSOC1* is also regulated by EARLY FLOWERING 9 (*ELF9*) and the gibberellic acid pathway (Song *et al*., 2009). Our data demonstrated an important role of *BdSOC1-like* transcription factors in regulating the shift from vegetative growth to inflorescence meristem identity. Plants overexpressing *BdSOC1-like* exhibited earlier flowering that might be correlated with increased *BdVRN1* and *BdLFY* expression, which suggests that *BdSOC1-like* might control the shift from vegetative growth to inflorescence meristem identity by upregulating the expression of *BdVRN1* and *BdLFY* (Fig. 3). On the contrary, the overexpression of *BdSOC1-like* caused the seeds to exhibit defective characteristics, which was consistent with the hypothesis that the downregulation of the *BdSOC1-like* gene was essential for seed development (Fig. 4).

Conclusion

In our experiment, *BdSOC1-like was* isolated and characterized using homology-based cloning. It encodes an

ORF of 684 bp and a putative protein of 227 amino acids. Bioinformatics analysis suggested that *BdSOC1-like* contained MADS-box (91.93% identity), K-box, C-terminal domain and SOC1 motif, and was a member of the MADS-box family. In our study, the expression of *BdSOC1-like* was widely detected in different tissues and organs at various growth stages. Also, qRT-PCR analysis found that the accumulation of *BdSOC1-like* gene did not display a diurnal rhythm under LD or SD conditions, and its expression in Bd21 did not appear to remember the prolonged cold treatment.

Furthermore, the promotion of flowering by the overexpression of *BdSOC1-like* in Bd21 suggested that *BdSOC1-like* might be active during the floral transition. The overexpression of *BdSOC1-like* also caused the seeds to exhibit defective characteristics. Together, above results enhance our understanding of the flowering mechanism in Bd21.

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